ThermoFisher SCIENTIFIC

HPLC columns

ProPac 3R SAX 3 µm columns

Product manual

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Contents

Introduction

Strong anion exchange technology

Thermo Scientific™ ProPac™ 3R Strong Anion Exchange (SAX) 3 µm columns are designed to provide fast, high efficiency, high resolution separations of acidic proteins and associated variants and adeno-associated viruses (AAVs) based on their accessible surface charge. The 3 µm, monodisperse particle is non-porous and based on a divinylbenzene resin coated with a hydrophilic polymer layer to exclude proteins from the surface of the resin and minimize secondary interactions. Grafting of quaternary amine functional groups to the hydrophilic surface introduces permanently charged cationic sites to provide the strong anion exchange character required for promoting protein and AAV binding when using a low ionic strength mobile phase at an appropriate pH. Running a gradient from low to high ionic strength mobile phase or from high to low pH disrupts the ionic protein and AAV-surface interactions resulting in elution of the sample and associated variants based on their relative strength of interaction with the surface.

SAX chromatography applications

SAX column technology is widely used in biopharmaceutical development and qualification of protein-based therapeutics. Therapeutic proteins have been used as a major class of treatment for various diseases including cancer, cardiovascular diseases, and autoimmune disorders, and continue to grow as therapeutics become more complex. Proteins typically have an isoelectric point (pI) ranging from 4.0 to 12.0, from being highly acidic to extremely basic. SAX columns in particular are used for the evaluation of acidic proteins (e.g., pI ≤ 7.0) as a result of their amino acid profile, glycosylations, and other post-translational modifications. Many acidic proteins such as circulatory proteins are often heavily glycosylated resulting in very complicated variant profiles.

This manual gives guidance on column use and care and demonstrates an approach to designing methods for the evaluation of acidic proteins and AAVs using an SAX column. Practical examples of method design are shown and discussed for the development of both a fast QC method and a longer high-resolution analytical method for Protein G and associated variants. Protein G was selected as a model protein in this case because it has a pI value of 4.5 and a complicated variant/impurity profile.[1] Method development examples for AAV analysis are also provided.[2] As the complexity of therapeutics increases, continued improvements in analytical technologies will be required to characterize these biomolecules and fulfill regulatory requirements required to bring them to market.

Introduction Continued

ProPac 3R SAX 3 µm column

The ProPac 3R SAX has a unique resin with a hydrophilic coating and quaternary ammonium groups. The packing material is based on a 3 µm, nonporous, divinylbenzene monodisperse polymer particle shown in Figure 1. Both the chemistry and size of the base monodisperse particle are tightly controlled. The chemistry consistency provides a solid platform for reproducibly producing the hydrophilic layer and quaternary amine SAX functionality using controlled polymerization techniques. The 3 µm monodisperse particle enables shorter diffusion distances resulting in more reproducible mass transfer and narrower peaks. Due to increased capacity associated with smaller particles, shorter columns can also be used for shorter run times with improved separation relative to larger particle media that require longer column lengths to achieve the same separation.

The uniform size of the particles enable precise control over the column packing. The ProPac 3R SAX media are packed in PEEK (polyether ether ketone) hardware, which has well-established bioinert properties to minimize nonspecific adsorption of protein samples.[3] The reproducible resin size and chemistry combined with controlled synthetic and packing manufacturing processes provide excellent lot-to-lot and column-to-column reproducibility.

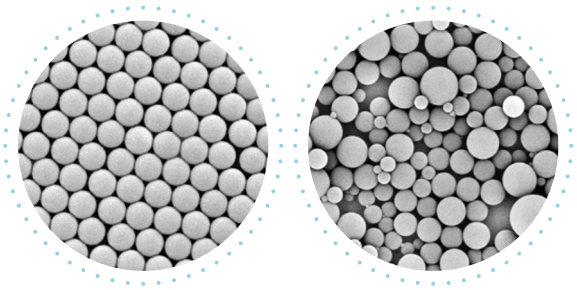


Figure 1: SEM image of 3 µm monodisperse ProPac 3R particles (left) vs. 3 µm polydisperse particles (right)

Table 1. ProPac 3R SAX 3 µm columns

Description	Dimensions	Particle size	Cat. no
Thermo Scientific ProPac 3R SAX columns	2 mm x 50 mm	3 µm	43203-052068
	2 mm x 100 mm	3 µm	43203-102068
	4 mm x 50 mm	3 µm	43203-054068
	4 mm x 100 mm	3 µm	43203-104068

Operation: System requirements and column use and care

Getting started

Prior to using the ProPac 3R SAX 3 µm column, review all the information in this section on system requirements and column operation. Following these specifications for your system and column will help to ensure the column performs as it is intended and maximize the lifetime of your column.

System requirements

The ProPac 3R SAX 3 µm columns are designed to be used with a standard bore bio-inert HPLC or UHPLC system having a gradient pump module, autosampler, injection valve, and a detector appropriate for your application (UV, DAD, Fluorescence, MS). A Thermo Scientific™ Vanquish™ Flex, Thermo Scientific™ Vanquish™) Horizon, or Thermo Scientific™ UltiMate™ 3000 BioRS totally inert UHPLC system is recommended for best results. A properly setup system is required to ensure good chromatographic performance and to extend the lifetime of your column.

A biocompatible system is highly recommended for mobile phases containing halide salts that can cause corrosion of metallic components. Metal leaching from the system components including the pump and connection tubing can contaminate the column and compromise chromatographic performance. Avoid using stainless steel tubing, ferrules, and bolt assemblies and replace them with MP35N tubing, PEEK, or other equivalent inert alternatives. For column-to-tubing connections, Thermo Scientific™ Viper™ Capillary MP35N with Thermo Scientific™ Viper™ Connection Fittings are strongly recommended. If your column has become contaminated with metals, the performance can be recovered by flushing with an EDTA (ethylenediaminetetraacetic acid) solution as described in Table 3.

System void volume

Tubing between the injection valve and detector should be ≤ 0.130 mm (0.0050” ID) tubing to minimize dispersion. Minimize the length of all liquid lines, especially the tubing between the column and the detector. The use of larger diameter and/or longer tubing may decrease peak efficiency and peak resolution.

Column use and physical specifications

To ensure that you do not damage the column hardware or packed bed, take care to operate within the limits of the column. Table 2 indicates the operational limits for each column format in terms of flow rate, maximum column pressure drops from inlet to outlet, temperature, and mobile phase pH.

Table 2. Recommended column operating conditions for optimal performance and extending column lifetime

Column	Flow rate (mL/min)	Max column pressure drop[1] psi (bar)	Temp °C	pH
2 mm x 50 mm	0.1-0.2	4500[2] (310)[2]	Ambient – 60°C	2-12
2 mm x 100 mm				
4 mm x 50 mm	0.3-0.5			
4 mm x 100 mm				

[1]The column pressure drop for a given flow rate is calculated as the pressure of the system with column minus the pressure of system with union in place of column
[2]For PEEK body columns, the maximum pressure at the column inlet should not exceed 7000 psi (485 bar) to avoid damaging the column body

Additional requirements for safe column operation:

- Always set up the mobile phase flow direction as indicated on the column tag. Flowing mobile phase opposite the intended direction will cause irreversible column damage
- Avoid sharp pressure fluctuations as they may disrupt the column bed
- When starting, stopping, or changing the flow rate, a flow ramp rate (mL/min/min) of ~1/3 of the maximum flow rate for the specific column format is recommended

Operation: System requirements and column use and care Continued

Recommended buffers for salt gradient separations

Salt gradient separations typically offer the best resolution possible for individual applications. Please consult Table 3 below for recommended buffer conditions to achieve optimal separations and maintain good column performance throughout its lifetime.

Table 3. Recommend buffers and mobile phase requirements

Parameter	Recommended
Buffer	• Tris or other Good's buffers • LC-MS and pH gradient: Ammonium acetate, ammonium bicarbonate, ammonium formate and associated acids and bases for pH gradients[3]
Minimum salt concentration	• 20 mM NaCl to avoid high pressure that can damage the column stationary phase • **Caution:** Never use pure deionized water on the column as this will result in irreversible damage
Detergent additives	• Nonionic, cationic or zwitterionic detergents • **Caution:** Do not use anionic detergents as they will irreversibly bind to the column and reduce the separation power
Organic solvent compatibility[4]	• Up to 20% acetonitrile • Up to 10% methanol
Cleaning agents	• For metal contamination (Fe, Cu, etc.) removal, flush the column at 0.4× the max column flow rate for 12 hours with 10 mM EDTA + 50 mM NaCl adjusted to pH 8
Storage solution	• Short term: ≥ 20 mM NaCl and your application buffer • Long term: ≥ 20 mM NaCl and your application buffer + 0.1% sodium azide

[3]Due to the weak ionic strength of volatile pH buffers, use lower flow rates for initial method development until the column back pressure is understood. The flow rate can then be increased as needed while still observing the maximum allowed pressure for the column

[4]Acetonitrile and methanol have viscosity maxima when mixed with water at certain ratios. This may cause unexpectedly high pressure. Always use low flow rates until the pressure behavior is understood when using these chemicals. Mixtures of ACN and MeOH should be introduced and removed gradually from the column using a gradient over 20 minutes to ensure a sharp viscosity front does not result in a rapid pressure difference in-column that may damage the packed bed

Recommended buffers for pH gradient separations

Volatile buffers can be used as indicated in Table 3 for pH gradient and LC-MS applications. Since very low ionic strength buffers can result in high column backpressures, begin your method development using low flow rates until the column pressure is understood with your selected mobile phase. Once the pressure conditions are understood, the flow rate can be scaled as needed while continuing to operate within the "Max Column Pressure Drop" indicated in Table 2.

Column conditioning

Your column has been designed to minimize secondary interactions and for low carryover. Depending on the nature of your protein sample, column conditioning may be required prior to achieving optimal performance. To quickly condition your column, we recommend performing 1-2 sample overload injections of 10× your standard protein sample loading and standard gradient method.

Minimum equilibration volumes for buffers

Prior to sample loading and start of the gradient, the column solid phase must be properly equilibrated to the loading conditions to promote binding of the analytes. Salt and pH gradient buffers require different volumes of buffer at sample loading conditions to equilibrate the column. If using a buffer system other than the ones recommended above, it is strongly advised that the user evaluate the required equilibration volume by examining the UV or pressure trace of the mobile phase when making a step change to their preferred loading conditions. The volume of mobile phase eluted from the time of the step change to when steady state is observed for the mobile phase UV or pressure trace represents the minimum volume required for equilibration of the stationary phase.

Operation: System requirements and column use and care Continued

Column storage and extended care

To maintain the performance of your column between uses, always store the column filled with the recommended buffers as detailed in Table 3. Use the plugs the column was shipped with to seal the ends of the column to prevent evaporation of the buffer and drying of the stationary phase. The formation of salt crystals in a dried bed may result in column clogging or reduced column performance in subsequent uses.

Depending on the length of time between column uses, the storage buffers should be used as follows:

- Short term storage (≤1 day): Fill the column with a low ionic strength buffer (e.g., 20 mM Tris + ≥20 mM NaCl)
- Long term storage (>1 day): Fill the column with a low ionic strength buffer + ≥20 mM NaCl and supplemented with 0.1% sodium azide

Sodium azide serves as a preservative to prevent bacterial growth that can damage the column phase, resulting in column clogging, and decreased column performance. When using the column again after storage with a sodium azide containing buffer, flush the column with ≥ 10 column volumes of buffer to remove all the sodium azide before starting any sample runs. At least 2 blank runs with a gradient from low to high salt concentration prior to any sample runs are also recommended for best performance and to establish a stable baseline for the initial sample runs.

Column performance verification

Each column is shipped with two Certificates of Analysis (CoA) one verifying the resin performance and one verifying the column performance. The resin qualification CoA includes a salt gradient separation of Protein G at 5 mg/mL, and the column qualification CoA shows an isocratic test of iodate ion. Each CoA provides the test conditions used. These tests can be reproduced to check the performance of your column. Note that differences in system configuration may result in differences in retention time and chromatographic performance. Table 4 provides purchasing information for the samples used in the CoA tests.

Table 4. Samples used for resin lot qualification and column performance testing

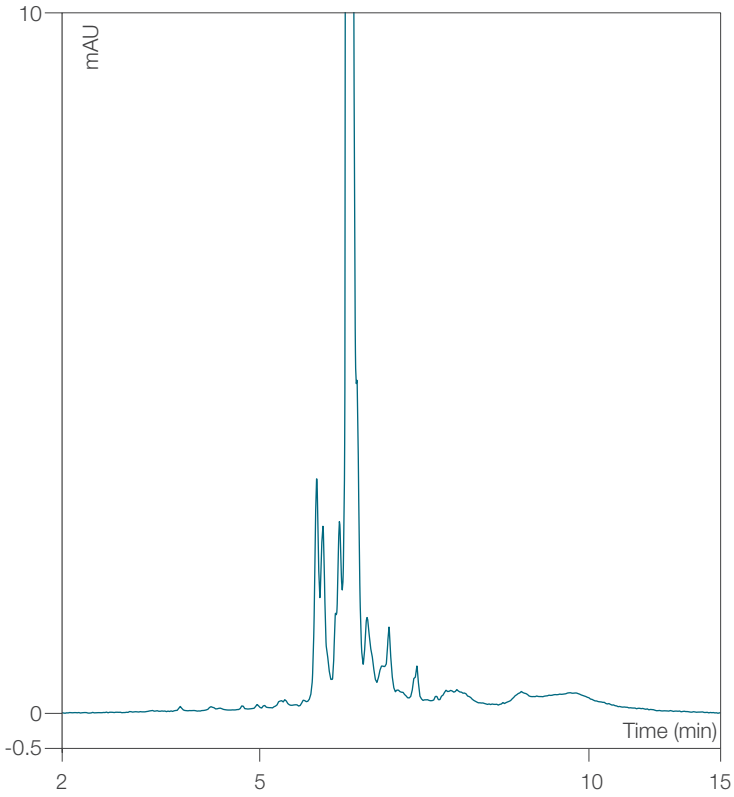
Sample	Supplier	Cat. no.
Protein G	Pierce	**77675**
Sodium Iodate	Sigma Aldrich	**S4007**

SAX chromatography applications

SAX chromatography applications – Protein G

Salt gradient separation is a common approach used for protein and variant analysis on SAX columns. The protein is first loaded onto the column stationary phase using a low ionic strength buffer. The ionic strength of the solution is increased over time typically using a salt such as NaCl. At higher salt concentrations, the anionic salt component will exclude the interactions of the anionic protein species from the quaternary ammonium groups of the stationary phase leading to protein desorption and elution from the column. Proteins with a greater number of anionic groups and/or fewer cationic groups will tend to elute at higher salt concentrations relative to those with fewer anionic groups and/or more cationic groups.

Salt gradients can provide excellent separation of proteins and their associated variants; however, these methods do require considerable development time investigating different buffer components, pH, salts, gradient slopes, temperatures, etc. For a comprehensive method development of Protein G using a salt gradient with a 4 x 100 mm, 3 µm ProPac 3R SAX column, please refer to application note “Salt gradient analysis of Protein G using a 3 µm monodisperse SAX chromatography column”.[1]



Column	ProPac 3R SAX, 3 µm		
Format	4 × 100 mm		
Mobile phase	A: 20 mM Tris, pH 8.0 B: 20 mM Tris + 500 mM NaCl, pH 8.0		
Flow rate	0.5 mL/min		
Injection	1 µL		
Temp	30 °C		
Detection	UV, 280 nm		
Sample	Protein G – 5 mg/mL		
Gradient		**%A**	**%B**
Time (min)	0.0	88	12
	6.0	58	42
	6.1	0	100
	8.0	0	100
	8.1	88	12
	20.0	88	12

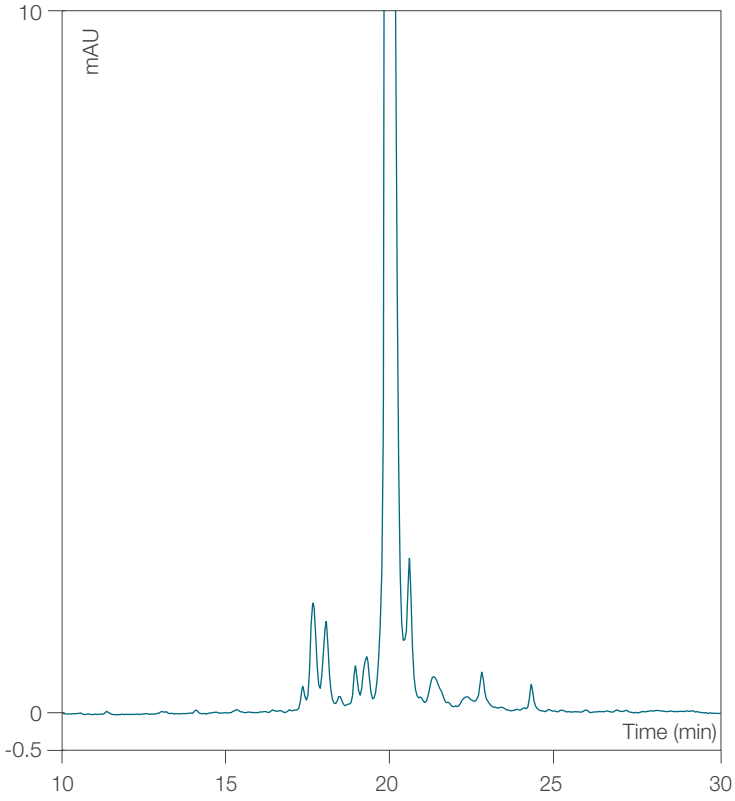
Figure 2: Chromatogram of a fast method with a 6 min gradient at 0.5 mL/min flow rate

SAX chromatography applications Continued

Optimized salt gradient methods – short vs. long

The ProPac 3R SAX column can be used for both rapid analytical methods and longer higher resolution methods. Figure 2 shows a fast method using a 6 min gradient at 0.5 mL/min flow rate to analyze protein G. By contrast Figure 3 uses a longer high-resolution analytical method with a 30 min gradient at 0.3 mL/min flow rate to achieve better resolution of each variant.

The high resolution and capacity of the ProPac 3R SAX column provides narrow peaks with sufficient retention time separation to detect the large number of variants associated with Protein G in a short amount of time. The longer gradient approach is suitable for explicit quantitation of peaks and/or fraction collection experiments in which the user wishes to analyze the variants in greater detail using orthogonal chromatography methods or assays. The flexibility of use and robust range of operating conditions for the ProPac 3R SAX column enables the user to design methods for a wide range of applications.



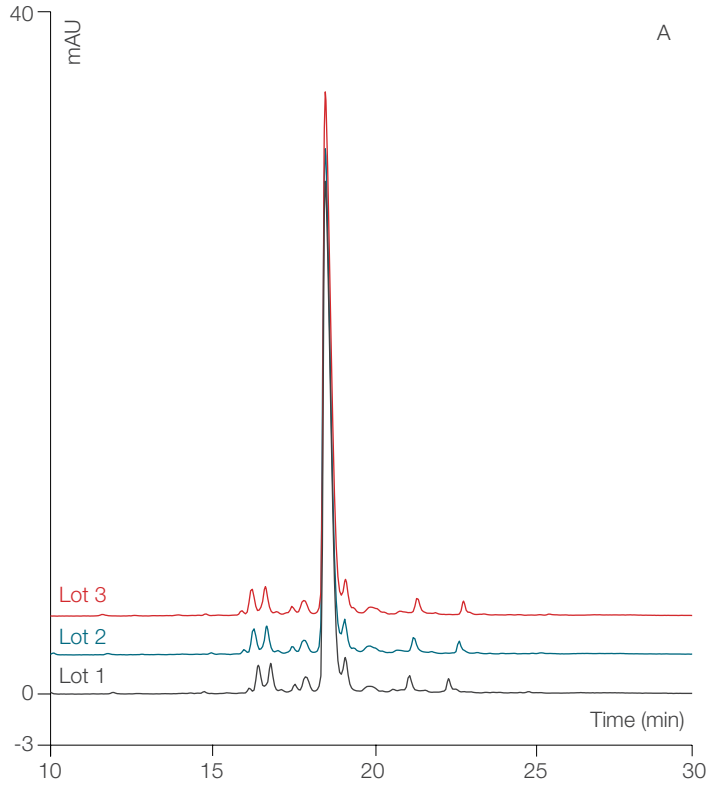
Column	ProPac 3R SAX, 3 µm		
Format	4 × 100 mm		
Mobile phase	A: 20 mM Tris, pH 8.0 B: 20 mM Tris + 500 mM NaCl, pH 8.0		
Flow rate	0.3 mL/min		
Injection	1 µL		
Temp	30 °C		
Detection	UV, 280 nm		
Sample	Protein G – 5 mg/mL		
Gradient		**%A**	**%B**
Time (min)	0.0	88	12
	1.0	88	12
	31.0	58	42
	31.1	0	100
	33.0	0	100
	33.1	88	12
	45.0	88	12

Figure 3: Chromatogram of a longer high-resolution analytical method with a 30 min gradient at 0.3 mL/min flow rate

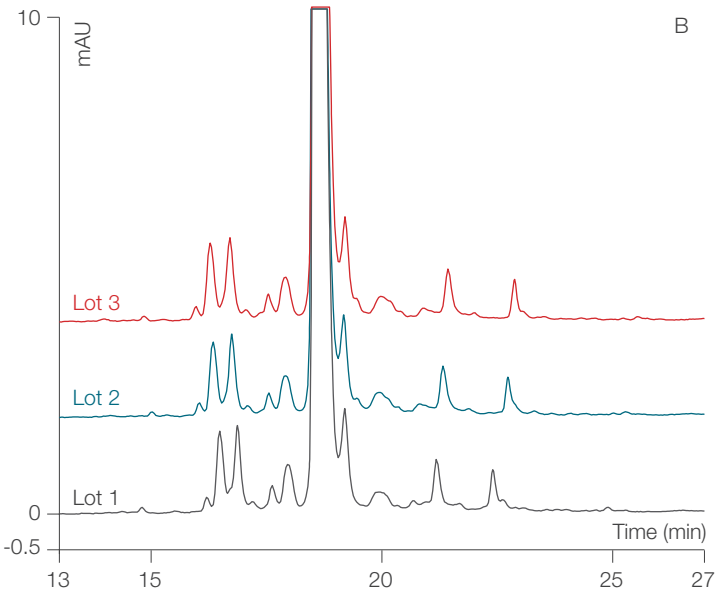
Column-to-column and lot-to-lot consistency is important for users to obtain reliable separations with every column. The ProPac 3R technology platform made using monodisperse particles and precision-controlled chemistry makes this possible.

Figure 4A shows the analysis of Protein G using the 30-minute gradient optimized method with columns from three different lots of media, with Figure 4B providing an enlarged view at 15 to 25 min. Here we can see robust performance providing excellent reproducibility lot-to-lot to give the same, reliable separation profile for chromatographers.

SAX chromatography applications Continued



Column	ProPac 3R SAX, 3 µm		
Format	4 × 100 mm		
Mobile phase	A: 20 mM Tris, pH 8.0 B: 20 mM Tris + 500 mM NaCl, pH 8.0		
Flow rate	0.5 mL/min		
Injection	1 µL		
Temp	30 °C		
Detection	UV, 280 nm		
Sample	Protein G – 5 mg/mL		
Gradient		**%A**	**%B**
Time (min)	0.0	88	12
	1.0	88	12
	31.0	58	42
	31.1	0	100
	33.0	0	100
	33.1	88	12
	45.0	88	12



Column	ProPac 3R SAX, 3 µm		
Format	4 × 100 mm		
Mobile phase	A: 20 mM Tris, pH 8.0 B: 20 mM Tris + 500 mM NaCl, pH 8.0		
Flow rate	0.5 mL/min		
Injection	1 µL		
Temp	30 °C		
Detection	UV, 280 nm		
Sample	Protein G – 5 mg/mL		
Gradient		**%A**	**%B**
Time (min)	0.0	88	12
	1.0	88	12
	31.0	58	42
	31.1	0	100
	33.0	0	100
	33.1	88	12
	45.0	88	12

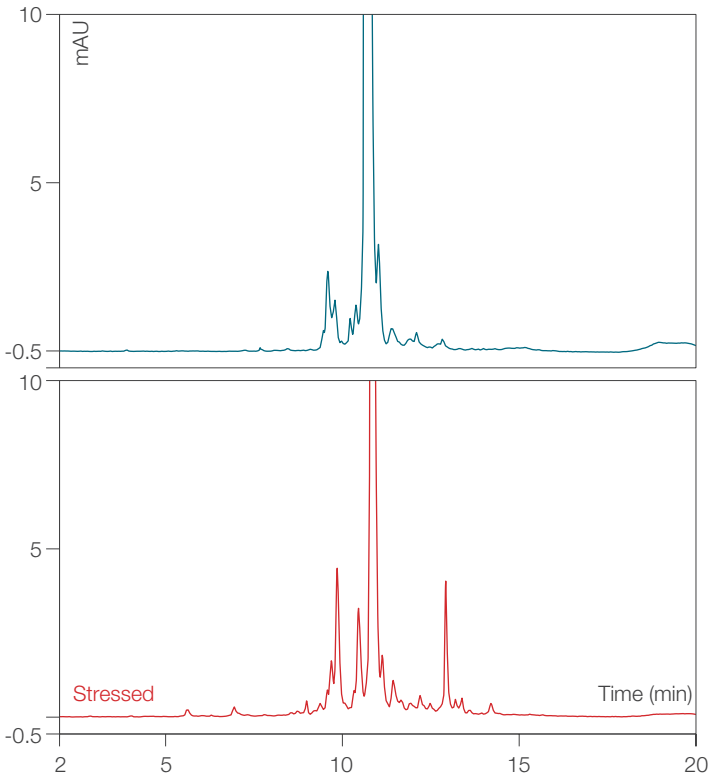
Figure 4: (A) Chromatograms of three different lots of ProPac SAX columns with a 30-minute gradient at 0.3 mL/min flow rate and (B) an enlarged view of the chromatograms. The retention time and signal of the main peak are normalized for ease of comparison of variant separation.

SAX chromatography applications Continued

Stressed sample evaluation

To demonstrate the utility of the ProPac 3R SAX column, we provide a practical example evaluating Protein G in its native form against a Protein G sample that has been stressed at 40 °C for 72 hours to observe modifications associated with thermal stress. Figure 5 compares these unstressed and stressed Protein G samples.

The temperature treatment of the protein results in the presence of more acidic and basic variants with some of the pre-existing variants significantly increasing in abundance. Despite the increase in variants for the stressed sample, the ProPac 3R SAX column maintains excellent resolution of the peaks, enabling easy comparison against the unstressed sample.



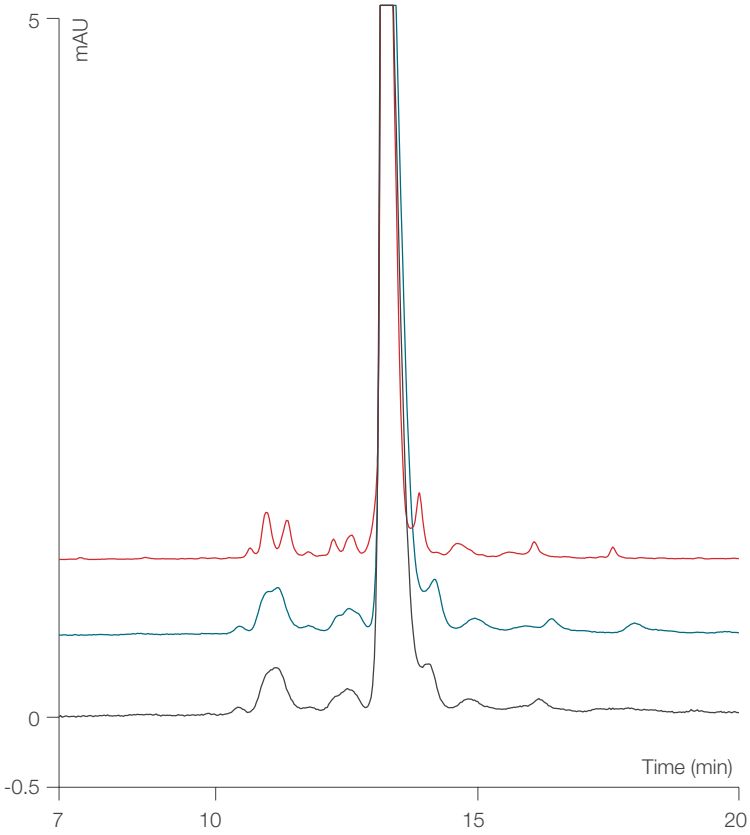
Column	ProPac 3R SAX, 3 µm		
Format	4 × 100 mm		
Mobile phase	A: 20 mM Tris, pH 8.0 B: 20 mM Tris + 500 mM NaCl, pH 8.0		
Flow rate	0.5 mL/min		
Injection	1 µL		
Temp	30 °C		
Detection	UV, 280 nm		
Sample	Protein G – 5 mg/mL		
Gradient		**%A**	**%B**
Time (min)	0.0	88	12
	1.0	88	12
	16.0	58	42
	16.1	0	100
	18.0	0	100
	18.1	88	12
	30.0	88	12

Figure 5: Chromatograms of non-stressed (top) and stressed (bottom) Protein G samples with a 15-minute gradient at a 0.5 mL/min flow rate

Lastly, we provide a comparison with ProPac SAX-10 columns in Figure 14 to demonstrate the superiority of the new ProPac 3R SAX column. For the ProPac SAX-10 column, the flow rate is increased to 1 mL/min to maximize performance and the injection volume is scaled to the column length. For direct comparison of the media for each product, a custom 4 × 100 mm ProPac SAX-10 column (black trace) was packed for this evaluation.

The 4 × 250 mm format (blue trace) is also provided to show the superior performance of the ProPac 3R SAX column despite its shorter length of 100 mm. The results show the significant advantage of the ProPac 3R SAX column (red trace) in the protein and charge variants separation, with narrower peaks and a greater number of basic and acidic variants being detected and resolved.

SAX chromatography applications Continued



Columns	Black: ProPac SAX-10, 10 µm, 4 ×100 mm Blue: ProPac SAX-10, 10 µm, 4 × 250 mm Red: ProPac 3R SAX, 3 µm, 4 × 100 mm		
Mobile phase	A: 20 mM Tris, pH 8.0 B: 20 mM Tris + 500 mM NaCl, pH 8.0		
Flow rate	Black: 1.0 mL/min Blue: 1.0 mL/min Red: 0.3 mL/min		
Injection	Black: 1 µL Blue: 1 µL Red: 2.5 µL		
Temp	30 °C		
Detection	UV, 280 nm		
Sample	Protein G – 5 mg/mL		
Gradient		**%A**	**%B**
Time (min)	0.0	88	12
	1.0	88	12
	31.0	58	42
	31.1	0	100
	33.0	0	100
	33.1	88	12
	45.0	88	12

Figure 6: Chromatograms of Protein G using a custom packed ProPac SAX-10, 10 µm 4 × 100 mm column (black), ProPac SAX-10, 10 µm 4 × 250 mm column (blue), and a ProPac 3R SAX, 3 µm 4 × 100 mm column (red) separately. The retention time and signal of the main peak are normalized for ease of comparison of variant separation.

SAX chromatography applications Continued

SAX chromatography applications – AAV

Adeno-associated viruses (AAV) are small replication-defective, nonenveloped viruses that can be used as vectors for gene therapy. Anion exchange (AEX) chromatography is commonly used to characterize AAV particles as both the empty and full capsids typically have a negative surface charge. Empty capsid typically has a higher pI (~0.4 pH units on average) than full capsid which may be due in part to full capsid being loaded with negatively charged DNA.[2]

Because AAV analysis are often sample limited, a 2 × 50 mm column and a fluorescence detector (FLD) were used for these separations. The 2 mm column ID was chosen because of the increased detection sensitivity provided by running at low flow rates when using low sample mass loading. The high capacity of the 3 µm ProPac 3R SAX media enables the use of a short 50 mm column format without sacrificing separation performance. The FLD detector was chosen over a variable wavelength detector due to the greater sensitivity of fluorescence-based detection techniques when using limited sample quantities. Here, we present a few examples of AAV separation. For a detailed discussion of method development for AAV samples, please refer to application note "Salt gradient separation and analysis of adeno-associated virus samples using a 3 µm monodisperse strong anion exchange chromatography column".[3]

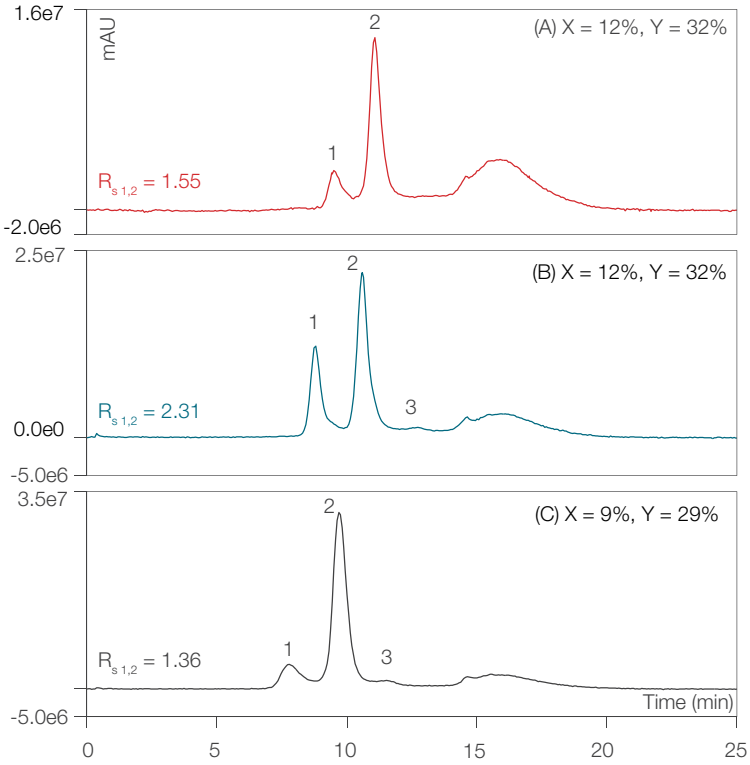
Linear salt gradients results

Figure 7 shows the analysis of AAV1, AAV6, and AAV8 samples using a simple linear salt gradient. Each AAV sample was created by mixing stock empty and full capsid samples in a 1:10 ratio. For each of the AAV samples tested, empty (peak 2) and full (peak 1) capsid peaks were baseline resolved using the linear gradient indicating that a simple linear salt gradient is often sufficient for separating these peaks for the purposes of quantitation. For AAV6 and AAV8 samples, an impurity peak (peak 3) was observed to elute after the full capsid peak.

For the AAV separations, the relative peak areas for empty capsids (peak 2) compared to full capsids (peak 1) are visually greater than 10% as expected based on the 1:10 mixing of empty:full standards. This is particularly obvious for AAV6 and may be due in part to the accuracy of sample concentrations from the supplier. However, we note that the fluorescence signal response for empty and full capsids is not equivalent, which will contribute to the differences in relative peak areas measured.[4] Calibration curves for both empty and full capsids would be needed to accurately measure the exact amounts of each capsid. For simplicity in this Application Note, we report the relative peak areas for empty and full capsids in subsequent analyses.

Using the spiked AAV6 sample, the lot-to-lot reproducibility for three different lots of ProPac 3R SAX media was evaluated using the linear gradient as shown in Figure 8. Good lot-to-lot reproducibility with baseline separation for the spiked AAV6 sample is observed for each lot.

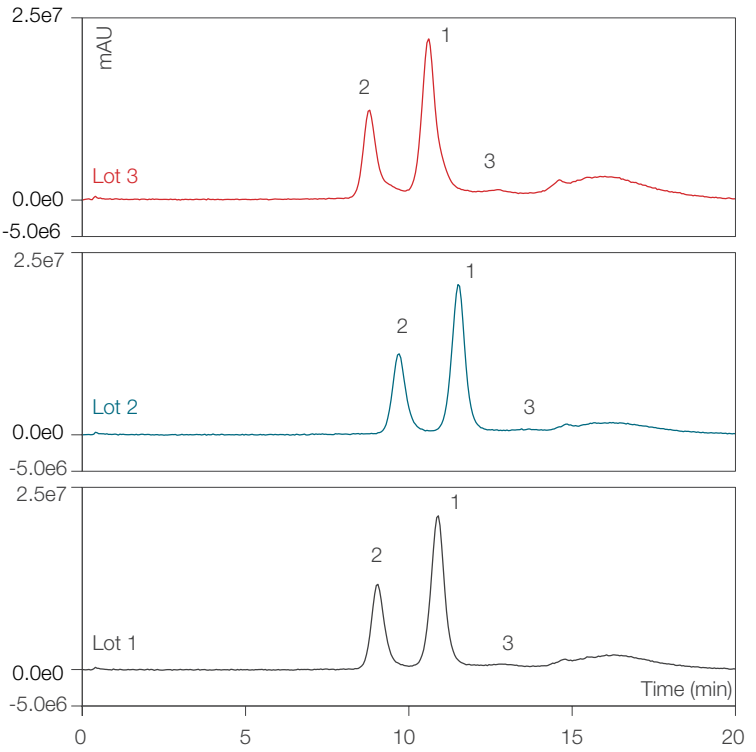
SAX chromatography applications Continued



Column	ProPac 3R SAX, 3 µm
Format	2 × 50 mm
Mobile phase	A: Water B: 1 M tetramethylammonium chloride C: 200 mM Bis-Tris Propane, pH 9.0
Flow rate	0.2 mL/min
Injection	0.2 µL
Temp	20 °C
Detection	FLD (Ex: 280 nm, Em: 330 nm
Sample	Concentration: 2 X 10^{13} vg/mL A: AAV1, Empty:Full 1:10 B: AAV6, Empty:Full 1:10 C: AAV8, Empty:Full 1:10
Peaks	1. Full capsid 2. Empty capsid 3. Impurity

Gradient		%A	%B	%C
Time (min)	0.0	90-X	X	10
	1.0	90-X	X	10
	11.0	90-Y	Y	10
	11.1	0	90	10
	13.0	0	90	10
	13.1	90-X	X	10
	25.0	90-X	X	10

Figure 7: Linear salt gradient separation of full capsid AAV samples spiked with empty capsid to give a 1:10 Empty: Full ratio: (A): AAV1 sample, (B): AAV6 sample and (C): AAV8 sample



Column	ProPac 3R SAX, 3 µm
Format	2 × 50 mm
Mobile phase	A: Water B: 1 M tetramethylammonium chloride C: 200 mM Bis-Tris Propane, pH 9.0
Flow rate	0.2 mL/min
Injection	0.2 µL
Temp	20 °C
Detection	FLD (Ex: 280 nm, Em: 330 nm
Sample	Concentration: 2 X 10^{13} vg/mL AAV6, Empty:Full 1:10
Peaks	1. Full capsid 2. Empty capsid 3. Impurity

Gradient		%A	%B	%C
Time (min)	0.0	78	12	10
	1.0	78	12	10
	11.0	58	32	10
	11.1	0	90	10
	13.0	0	90	10
	13.1	78	12	10
	25.0	78	12	10

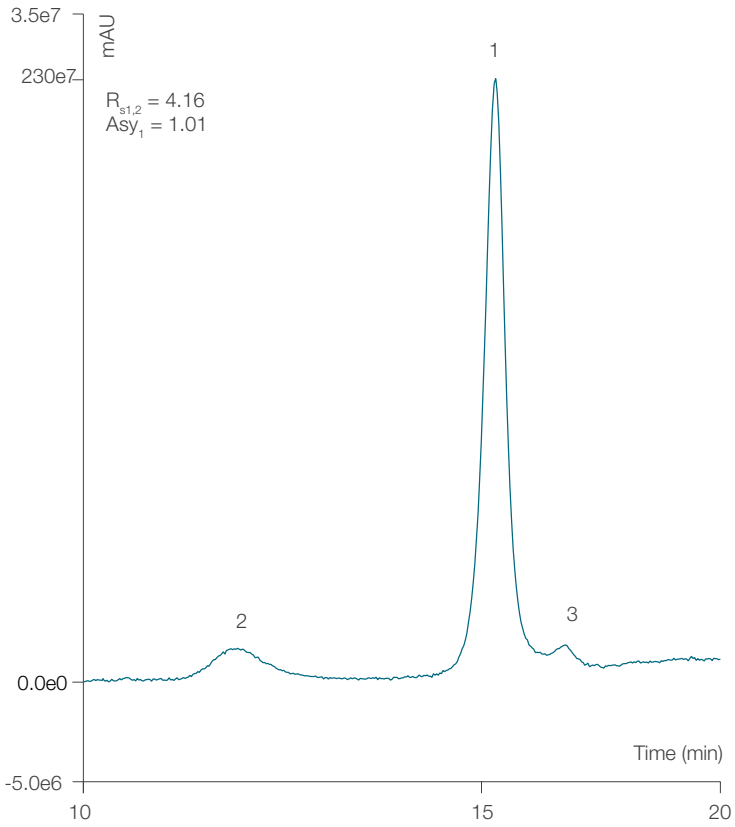
Figure 8: Separation of AAV6 sample on 3 different lots of ProPac 3R SAX media using the linear gradient shown in Figure 8.

SAX chromatography applications Continued

Linear salt gradient with isocratic hold method development and results

The simple linear salt gradient method is straightforward to implement and provides good separation for each of the AAV samples evaluated. By incorporating an isocratic hold, the separation of the empty capsid from the full capsid can be increased significantly. Figure 9 shows the results of 17% MPB for the isocratic elution stage with a 4 min hold.

Baseline separation of empty and full capsids is achieved with a resolution of 4.16 and the peak asymmetry is close to 1 (Asy_1 = 1.01). The user can employ orthogonal methods such as AUC or cryo-EM[5] in combination with AAV standards to determine which %MPB for isocratic hold and isocratic holding time that will provide the most accurate characterization of the empty/full ratio.



Column	ProPac 3R SAX, 3 µm			
Format	2 × 50 mm			
Mobile phase	A: Water B: 1 M tetramethylammonium chloride C: 200 mM Bis-Tris Propane, pH 9.0			
Flow rate	0.2 mL/min			
Injection	0.2 µL			
Temp	20 °C			
Detection	FLD (Ex: 280 nm, Em: 330 nm			
Sample	Concentration: 2 X 10^{13} vg/mL AAV6, Empty:Full 1:10			
Peaks	1. Full capsid 2. Empty capsid 3. Impurity			
Gradient		**%A**	**%B**	**%C**
Time (min)	0.0	88	2	10
	1.0	88	2	10
	6.0	73	17	10
	10.0	73	17	10
	15.0	40	50	10
	15.1	0	90	10
	18.0	0	90	10
	18.1	88	2	10
	30.9	88	2	10

Figure 9: Salt gradient separation of AAV6 separation using an isocratic hold at 17% MPB with a 4 min hold

Reference

1. Ma, K.; Bechler, S., Salt gradient analysis of Protein G using a 3 µm monodisperse SAX chromatography column, Thermo Fisher Scientific, 2023
2. Aebischer, M. K.; Gizardin-Fredon, H.; Lardeux, H.; Kochardt, D.; Elger, C.; Haindl, M.; Ruppert, R.; Guillarme, D.; D'Atri V., Anion-Exchange Chromatography at the Service of Gene Therapy: Baseline Separation of Full/Empty Adeno-Associated Virus Capsids by Screening of Conditions and Step Gradient Elution Mode. International journal of molecular sciences 2022, 23, (20), 12332.
3. Ma, K.; Bechler, S., Salt gradient separation and analysis of adeno-associated virus samples using a 3 µm monodisperse strong anion exchange chromatography column, Thermo Fisher Scientific, 2023
4. Wang, C.; Mulagapati, S. H. S.; Chen, Z.; Du, J.; Zhao, X.; Xi, G.; Chen, L.; Linke, T.; Gao, C.; Schmelzer, A. E.; Liu, D., Developing an Anion Exchange Chromatography Assay for Determining Empty and Full Capsid Contents in AAV6.2. Molecular Therapy – Methods & Clinical Development 2019, 15, 257–263
5. Khatwani, S.L.; Pavlova, A.; Pirot, Zhu., Anion-exchange HPLC assay for separation and quantification of empty and full capsids in multiple adeno-associated virus serotypes. Molecular Therapy – Methods & Clinical Development 2021, 21, 548-558.

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